Expression of α - and β -Tubulin Genes during Development of Sea Urchin Embryos

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Mature unfertilized eggs of the sea urchin Lytechinus pictus contain multiple α -tubulin mRNAs, which range in size from 1.75 to 4.8 kb, and two β -tubulin mRNAs, 1.8 and 2.25 kb. These mRNAs were found at similar levels throughout the early cleavage stages. RNA gel blot hybridizations showed that prominent quantitative and qualitative changes in tubulin mRNAs occurred between the early blastula and hatched blastula stages. The overall amounts of α - and β -tubulin mRNAs increased two- to fivefold between blastula and pluteus. These increases were due mainly to a rise in a 1.75-kb α RNA and a new 2.0-kb β RNA. Other, minor changes also occurred during subsequent development. All size classes of α - and β -tubulin RNAs in early and late embryos contained poly(A)⁺ translatable sequences. As reported earlier, some of each of the α RNAs, but neither of the β RNAs, are translated in the egg and a small portion of each of the stored α and β RNAs is recruited onto polysomes within 30 min of fertilization. In the work described here, subsequent development up to the morula stage was accompanied by a gradual recruitment of tubulin mRNAs into polysomes. By the early blastula stage, most of the maternal tubulin sequences were associated with polysomes. In contrast to the gradual recruitment of maternal sequences throughout cleavage, the tubulin mRNAs which appeared at the blastula stage showed no delay in entering polysomes. The exact fraction of each mRNA that was translationally active at later stages varied somewhat among the individual mRNAs. From the differential hybridization patterns of egg, embryo, and testis RNAs to various tubulin cDNA and genomic DNA probes, it is concluded that at least one gene producing maternal α mRNA is different from a second one which is expressed only in testis. Each of the three embryonic β RNAs is encoded by a different β gene; at least two of these different β genes are also expressed in testis. © 1985 Academic Press, Inc.

INTRODUCTION

The developing sea urchin embryo requires tubulin for at least three different kinds of activities. Tubulin is needed to form the microtubules of the mitotic spindle in rapidly dividing cleavage-stage embryos (Raff *et al.*, 1971; Raff and Kaumeyer, 1973). It is also used to form the cilia of the swimming blastula-stage embryos (Auclair and Siegel, 1966; Stephens, 1972; 1977; Raff *et al.*, 1971). Finally, tubulin is assembled into microtubules which are involved in some of the changes in cell shape that accompany gastrulation (Gibbins *et al.*, 1969; Tilney and Gibbins, 1969).

The α - and β -tubulins are provided in the embryo from three different sources: a stockpile of proteins stored in the egg, a pool of "maternal" mRNA stored in the egg and activated after fertilization, and newly transcribed embryonic mRNAs. The mature, unfertilized egg contains a large pool of soluble tubulin monomers, accounting for about 1-5% of total egg protein (Raff, 1975; Pfeffer *et al.*, 1976). This pool provides

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more than 99% of the tubulin that is recruited into the first mitotic spindle (Bibring and Baxandall, 1977) and probably contributes to the spindles of subsequent cleavage divisions. These tubulins are synthesized and accumulated during oogenesis (Cognetti et al., 1977) and stored in the mature egg in some soluble, nonpolymerized state. The work of Suprenant and Rebhun (1983) suggests that this tubulin is competent for polymerization and that the postfertilization assembly into microtubules depends on the appearance of mitotic organizing centers or assembly-promoting factors. The egg tubulin pool also contains a fraction that is destined for recuitment into cilia at the blastula stage (Bibring and Baxandall, 1981). The amount assembled into the mitotic spindles and cilia accounts for only about onetenth of the total pool (Cohen and Rebhun, 1970; Stephens, 1972). The fate of the rest of the stored tubulins is unknown. Curiously, despite this apparent excess, additional tubulin is made soon after fertilization and continues to be synthesized throughout early development (Raff et al., 1971).

Raff *et al.* (1972) first demonstrated that the egg contains a pool of mRNA that is responsible for most of the tubulin synthesis in the early embryo. There is little or no tubulin synthesis in the mature egg, and

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after fertilization tubulin synthesis rises roughly in parallel with the overall rise in protein synthesis, becoming quite prominent in the 16-cell embryo and later stages (Raff, 1975). Tubulin mRNA levels, as measured by *in vitro* template activity, remain at a fairly constant, low level from fertilization to hatching and then increase a few fold, presumably due to the accumulation of new transcripts (Alexandraki and Ruderman, 1981). We have recently used cloned α - and β -tubulin cDNAs to demonstrate directly the presence of stored tubulin mRNAs in the mature sea urchin egg (Alexandraki and Ruderman, 1985). In that work, we encountered a surprising amount of heterogeneity and polymorphism in the α -tubulin RNAs, finding that the egg contains five to seven different size classes of α RNAs ranging from 1.75 to 4.2 kb in length and varying in number, size, and relative abundancy among different females. The β RNAs are less variable, there being only two or three size classes of RNA (1.8, 2.25, and 2.9 kb) in eggs of different females. In spite of the unexpectedly long sizes of some of these RNAs, all represent translatable mRNAs. Those experiments. however, were unable to distinguish which of these mRNAs represent products of different genes, polymorphic transcripts of alleles, or alternatively processed transcripts of single genes.

There is a great deal of uncertainty over the number of functional α - and β -tubulin genes in the sea urchin and the number of different proteins encoded by them. Also, it is not yet resolved whether different microtubular organelles such as spindles, cilia, and flagella are constructed using the same or different tubulins. Our earlier experiments showed that there are about 9-13 α - (and β -) tubulin DNA sequences in the genome of the urchin Lytechinus pictus but did not distinguish between functional genes and pseudogenes (Alexandraki and Ruderman, 1981). DNA sequence analysis of three testis partial-length β -tubulin cDNA clones revealed two different nonallelic polypeptide chains differing by only a single amino acid among the 100 carboxyterminal residues compared, despite extensive differences in the 3' untranslated portions of the three cDNAs. Analyses of the proteins themselves revealed up to four α (and β) isoelectric variants in the egg (Dietrich and Wilson, 1983; Suprenant and Rebhun, 1983). Comparisons of amino acid content and tryptic peptide maps of tubulins from eggs, cilia, and flagella suggest that there are α - and β -tubulins of different primary structure from each of these organelles (Stephens, 1978). Two different monoclonal antibodies produced from flagellar axonemes indicate heterogeneity of α -tubulins (Asai *et al.*, 1982). However, in all of these comparisons, post-translational modifications cannot be ruled out as sources of variation.

As part of our effort to understand the expression of different tubulin genes and their roles in early development, we have investigated the translational recruitment of the maternal tubulin mRNAs and of newly accumulated embryonic tubulin transcripts during embryonic development up to the pluteus stage. We found that the stored maternal tubulin mRNAs were gradually recruited onto polysomes between fertilization and the late morula stage. In contrast, new embryonic transcripts, which became prominent at the blastula stage, appeared to be loaded promptly onto polysomes. Using some different cDNA and genomic tubulin clones, we were able to correlate the expression of two α and three β genes with stage- and tissuespecific RNAs.

MATERIALS AND METHODS

Culture of sea urchin embryos. Gametes were collected from L. pictus (Pacific Biomarine, Venice, Calif.) following injection of 0.55 M KCl. Eggs were fertilized and cultured in 200 to 1000 vol of nitrocellulose membrane (0.45- μ m pore size, Millipore)-filtered seawater containing 10 μ g/ml gentamycin sulfate, with constant stirring at 16 to 18°C.

Preparation of RNA. For the extraction of total cellular RNA, eggs and embryos at selected stages of development were recovered by centrifugation (7 to 15 sec at 1600g), washed once with membrane-filtered seawater, twice with Ca^{2+} -, Mg^{2+} -free seawater (0.46 M NaCl, 11 mM KCl, 7 mM Na₂SO₄, and 10 mM Tris-HCl, pH 8), and once with homogenization buffer (0.35 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8). Cells were lysed in 3 to 5 vol of homogenization buffer containing 2% sodium dodecyl sulfate (SDS) and 7 Murea. The samples were deproteinized by repeated extractions with phenol:chloroform:isoamyl alcohol (25: 24:1) and then ethanol precipitated. In some experiments the RNA was purified from the extracted DNA by preferential precipitation in the presence of 2MLiCl at 4°C for 4 hr followed by centrifugation at 10,000g for 20 min.

For the preparation of cytoplasmic RNA, eggs and embryos were broken in 3 to 5 vol of ice-cold homogenization buffer containing 0.25% diethyl pyrocarbonate in a Dounce homogenizer and centrifuged at 15,000*g* for 15 min. An equal volume of SDS-urea buffer was added to the postmitochondrial supernatant fractions and each sample was deproteinized and then ethanol precipitated.

Testis cytoplasmic RNA and polyadenylic acid-containing RNA [poly(A)⁺ RNA] were prepared as previously described (Alexandraki and Ruderman, 1981). RNA samples were stored in H_2O at a concentration of 1 to 5 mg/ml (total cellular RNA) and 0.5 mg/ml [poly(A)⁺ RNA] at -80° C.

Preparation and fractionation of cytoplasmic postmitochondrial supernatants by density gradient centrifugation. The following procedures as similar to those described by Rosenthal *et al.* (1980, 1983). Eggs and embryos were homogenized in 2 to 3 vol of ice-cold homogenization buffer containing 0.3 M glycine, 250 mM KCl, 3 mM MgCl₂, and 40 mM Hepes, pH 7.3. Following centrifugation at 15,000g for 20 min, the postmitochondrial supernatant fractions were divided into small aliquots, frozen in liquid nitrogen, stored at -80°C, and used only once after thawing.

Thawed samples were centrifuged at 15.000a for 10 min and 100- to 200- μ l aliquots were diluted with 5 vol of cold high-salt gradient buffer (0.5 M KCl, 6 mM $MgCl_2$, 1 mM EDTA, and 10 mM Hepes, pH 7.4) and layered onto 11 ml of 15 to 40% sucrose gradients containing high-salt gradient buffer. These gradients were then centrifuged for 95 min at 40,000 rpm and 4°C in an SW41 rotor. In some experiments, mRNAs were released from polysomes prior to centrifugation by treatment with either puromycin or EDTA (Blobel, 1971; Young and Raff, 1979). When puromycin was used, 100- to $200-\mu$ l postmitochondrial supernatant fractions were brought up to 1-ml volume and made 0.5 M KCl, 3 mM MgCl₂, 1 mM EDTA, 40 mM Hepes, pH 7.5, and 2 mM puromycin dihydrochloride (Calbiochem) final concentrations. These samples were incubated for 15 min on ice and 15 min at 37°C. When EDTA was used, the samples were diluted in gradient buffer, made 30 mM EDTA, and incubated for 10 min on ice. Seven fractions were collected from each gradient directly into 5 ml of ice-cold ethanol and precipitated overnight in the presence of 5 μ g tRNA at -80° C. Pelleted samples were suspended in 0.5 ml of a solution containing 0.2 M LiCl, 2 mM EDTA, 2% SDS, and 0.1 M Tris, pH 7.6, extracted twice with 1 ml of phenol: chloroform: isoamyl alcohol (25:24:1), precipitated with ethanol, resuspended in 0.5 ml of H₂O, made 250 mM ammonium acetate, precipitated with ethanol, and finally dissolved in 10 μ l of H₂O and stored at -80°C until use.

Gel electrophoresis and blotting of RNA samples. RNA samples were made 20 mM Mops, 5 mM sodium acetate, 1 mM EDTA, pH 7, 50% formamide, and 2.2 M formaldehyde, incubated at 65°C for 5 min, and, following the addition of 14% glycerol and 0.02% tracking dyes, electrophoresed in 1.5% agarose gels containing 2.2 M formaldehyde and the Mops/acetate/EDTA buffer (Lehrach et al., 1977; Rave et al., 1979). Sea urchin 26 S (~3850 bases) and 18 S (1840 bases) rRNA and Escherichia coli 23 S (2904 bases), its specific breakdown products of ~1750 and ~1250 bases, and 16 S (1541 bases) were used as molecular weight standards. Restriction endonuclease fragments of λ , M13, and pBR322 DNAs were also used as molecular weight markers after initial denaturation by boiling and subsequent treatment similar to that of the RNA samples. Following electrophoresis the RNA was transferred onto nitrocellulose paper in 20× SSC for 12 to 18 hr (Thomas, 1980). The filters were then baked for 2 to 3 hr at 80°C in a vacuum oven, washed in 2× SSC, and pretreated, hybridized, washed, and autoradiographed following the procedures described by Wahl *et al.* (1979).

Preparation of DNA. Sea urchin sperm high-molecular-weight genomic DNA, bacterial plasmid DNA, and phage λ DNA were prepared as previously described (Alexandraki and Ruderman, 1981). Plasmid and phage DNA restriction endonuclease fragments that were used for subcloning experiments or as probes in DNA and RNA blotting experiments were isolated by gel electrophoresis and elution from 6 to 8% polyacrylamide Tris/borate/EDTA gels or by electroelution from 0.7-1% agarose gels (Maniatis *et al.*, 1982), depending on their size and their subsequent use.

Subcloning of DNA fragments was carried out according to the following procedure. pBR322 and pSP64 DNA were digested with the appropriate restriction endonucleases and dephosphorylated with intestinal alkaline phosphatase (Boehringer-Mannheim) (Maniatis et al., 1982). Plasmid (0.1 μ g) DNA was ligated with a two- or threefold molar excess of the DNA fragment to be subcloned by using T4 DNA ligase (New England Biolabs). Transformations of E. coli HB101 with the recombinant plasmids were performed by the calcium chloride procedure (Maniatis et al., 1982). The presence of recombinant DNA in bacterial colonies was confirmed by restriction endonuclease analysis of isolated plasmid DNAs and Southern blotting analysis. DNA probes were labeled with [³²P]dNTPs by nick-translation using DNA polymerase I (New England Biolabs) in the presence of deoxyribonuclease I, to specific activities up to 4×10^8 cpm/µg.

DNA blotting. DNA electrophoresis and transferring to nitrocellulose paper, followed by hybridization and autoradiography of filters, were carried out as previously described (Alexandraki and Ruderman, 1981).

RESULTS

Stage-Specific α-Tubulin mRNAs in the Sea Urchin Embryo

Total and cytoplasmic RNAs isolated from unfertilized eggs and embryos at different developmental stages were analyzed for the presence of α - and β -tubulin transcripts by denaturing gel electrophoresis and filter hybridization with cloned cDNA and genomic α - and



FIG. 1. Autoradiograms of gel-blotted α -tubulin RNAs from different cultures of eggs and embryos at specific stages of development. I to VIII indicate the different cultures from which RNA was prepared. Cultures I, III, IV, V, and VII contained eggs pooled from three different females, cultures VI and VIII from two females, and culture II from one female. Aliquots (5 μ g) of total (I-VI) and cytoplasmic (VII and VIII) RNA isolated from eggs and embryos were electrophoresed in agarose gels, transferred onto nitrocellulose filters, and hybridized with ³²P-labeled G-p α 21B2 (A-D) and p α 2 (B'-D') DNAs. The developmental stages are indicated by the number of hours after fertilization, shown on the top line above panel A. The morphological stages corresponding to these times are given on the second and third lines. Lanes corresponding to stages indicated on the second line were prepared from samples of one culture; lanes corresponding to stages shown on the third line were prepared from another, different culture. The specific developmental stages in each set of cultures were mature unfertilized egg (e); 2-cell embryo (2c); 16-cell embryo (16c); early blastula (eb); blastula (b); rotating blastula (rb); swimming blastula (sb); late mesenchyme blastula (lmb); early gastrula (eg); prism (pr); and plutcus (pl). Aliquots (0.5 μ g) of poly(A)⁺ RNA isolated from the RNA samples shown in panels A and B gel-blotted and hybridized with G-p α 21B2 probe are displayed in panels E and F, respectively.

 β -tubulin probes. Because our initial analysis of egg tubulin mRNA showed significant polymorphism of α tubulin mRNAs and, to a smaller extent, of β -tubulin mRNAs in eggs from different individuals (Alexandraki and Ruderman, 1985), we analyzed several different embryonic cultures. Each of the four developmental series presented in Fig. 1 covers the first 3 days of development. Because there was not enough material in any one culture for all of the necessary time points, we used one culture for the early time points and a second, different culture for the late time points. The first culture spanned development from fertilization to the 6- to 9-hr blastula stage (I, III, V, VII); the second culture covered development from the blastula to the pluteus stage (II, IV, VI, VIII). Aliquots of eggs from both cultures and, when feasible, overlapping stages were also examined. In Fig. 1A, for example, culture I provided samples for egg, 2-cell, 16-cell embryo, early blastula, and swimming blastula RNA. Culture II was used for RNA samples from eggs, rotating blastula, swimming blastula, early gastrula, prism, and pluteus stages.

Two kinds of α -tubulin DNA probes were used. The first, $p\alpha 2$, is a testis-derived, cloned partial-length cDNA which has been completely sequenced; it contains 483 bases of coding and 300 bases of 3' untranslated sequences (Alexandraki and Ruderman, 1983). The $p\alpha 2$ hybridization patterns are shown on the right side of Fig. 1. The second probe, G- $p\alpha 21B2$, is a subcloned 1.5kb *Bgl*II fragment of the α -tubulin genomic DNA clone $\lambda LpT21$ (Alexandraki and Ruderman, 1981). Preliminary sequence data show that this fragment contains ~830 bases of the 3' portion of coding region, a termination codon, and 3' noncoding sequence within the remaining ~670 base pairs (data not shown). G- $p\alpha 21B2$ hybridization patterns are shown on the left side of Fig. 1.

As described earlier, the different egg preparations contained multiple α mRNAs: a faint 1.75-kb species and four or five more abundant size classes ranging from 2.2 to 3.6 kb (Alexandraki and Ruderman, 1985). The exact patterns varied among different egg RNA samples, with both the genomic and cDNA probes. In general, the genomic DNA probe G-p α 21B2 gave a stronger hybridization signal than did p α 2; moreover, it hybridized more intensely to the highest molecular weight mRNA in each sample. This is probably because it contains sequences specific for these transcripts.

Between fertilization and the early blastula stage (6 hr) these patterns remained essentially unchanged. Then all of the different cultures exhibited dramatic quantitative and qualitative changes. Unlike the polymorphism of the α -tubulin RNAs in the egg and early embryo, the α -tubulin RNA patterns of the later stages did not vary as much among the different cultures. The amount of the 1.75-kb RNA progressively increased, became the most abundant in the 17-hr hatched blastula, and remained at this high level up to the 67-hr pluteus larva. In parallel, the two largest egg α RNA classes (2.9-3.6 kb) decreased in amount and were undetectable after the 20-hr mesenchyme blastula. At that time, a new RNA of similar or slightly larger size (3.4-3.6 kb) appeared and remained thereafter. We think that this late-embryo RNA size class was newly accumulated because (1) it had a different size than those of the early RNAs (best seen in Figs. 1B and D) and (2) it showed higher hybridization signals than the early RNAs (which are G-p α 21B2 specific) with the $p\alpha 2$ probe (see Figs. 1B', C', and D'). The 2.65-kb mRNA, whose level varied widely in different cultures of early embryos, decreased progressively after the hatched blastula stage in all cultures and was barely detectable in the pluteus. A 2.9-kb RNA that was present only in culture VI showed a similar decline after the hatched blastula stage. The level of the 2.2kb RNA present in most early embryonic cultures decreased after the hatched blastula stage and increased again between 25 and 40 hr of development; this is best seen in Fig. 1B. During this same interval a 2.2-kb RNA appeared and increased in embryos derived from eggs that lacked this RNA (Fig. 1A, culture II). Finally, a 3.0- to 3.1-kb RNA was first detected in 40-hr prism embryos and became prominent in plutei. These changes in the less abundant late RNAs (>1.75 kb) can be seen better in the samples of polyadenylated RNAs (Figs. 1E and F), which were better resolved because they contained much less ribosomal RNA than those of total RNA.

Quantitation of the overall levels of α -tubulin RNAs from the different embryonic stages of cultures I and II showed that the amount of α -tubulin RNA increased after the mid-blastula stage; in later stages it was about two to five times higher than the level in cleavage stage embryos (Table 1). This increase was

hrs of development	cpm obtained with probe							
	pα2		G-pα21B2		pβ2		G-pβ6E2	
	I	II	Ι	II	Ι	II	I	II
0	49	52	480	537	178	142	180	210
1.5	47		572		150		207	
4	56		385		143		170	
6	73		405		162		178	
12.5		60 (1.1×)		482 (1×)		$198~(1.4 \times)$		183 (1×)
18		$162 (3.1 \times)$		1,043 (1.9×)		850 (5.9×)		560 (2.6×)
19	119 (2.1×)		865 (1.9×)		1,640 (10.3×)		427 (2.3×)	
26		153 (2.9×)		914 (1.7×)		$845~(5.9 \times)$		560 (2.6×)
41		215 (4.1×)		1,393 (2.6×)		1,035 (7.2×)		685 (3.2×)
67		$274 (5.2 \times)$		1,591 (3×)		1,597 (11.2×)		732 (3.5×)

TABLE 1 QUANTITATION OF THE RELATIVE AMOUNTS OF TOTAL α - and β -Tubulin RNAs during Development

Note. Aliquots (5 μ g) of LiCl-precipitated total RNA isolated from eggs and embryos of cultures I and II (see in Figs. 1A and 2A) were gel blotted and hybridized with ³²P-labeled α - and β -tubulin cDNA and genomic DNA probes. The total region containing autoradiographic bands in each lane of the RNA blots was cut out and counted by liquid scintillation counting. Counts per minute above background are given. Numbers in parentheses indicate the relative increase of cpm in samples from late embryos (>12.5 hr). The relative increase given in samples of culture I was calculated considering the average cpm number of 0- to 6-hr samples.

ization $(2-5\times)$ is more accurate than that obtained from G-p α 21B2 (2-3 \times) since only the coding sequence of p α 2 hybridizes to egg and embryo RNA whereas Gp α 21B2 contains sequences specific for early embryo RNAs, as discussed later. The increase in total α RNA in the blastula and its level at different late stages varied among the different cultures; however, all the values were in the same range of two- to fivefold increase (data not shown).

Accumulation of Different β -Tubulin mRNAs during Embryogenesis

The same egg RNA samples shown in Fig. 1 were hybridized with two β -tubulin DNA probes (Fig. 2). The testis cDNA $p\beta 2$ probe contains 392 bases of coding and 393 bases of 3' untranslated sequences (Alexandraki and Ruderman, 1983); the subcloned 2.8-kb EcoRI Gp β 6E2 fragment of the β -tubulin genomic clone λ LpT6 is described below. Both probes revealed two size classes of β transcripts, a prominent 2.25-kb RNA and a faint 1.8-kb RNA. In 2 of the egg RNA samples shown here (Fig. 2, cultures I and II) and in 1 of the 10 samples analyzed previously (Alexandraki and Ruderman, 1985), the 1.8-kb RNA was not detected. Also, none of the egg RNA samples shown here contained the 2.9-kb RNA seen in 3 of the 11 samples analyzed previously (Alexandraki and Ruderman, 1985). As in the case of α -tubulin RNAs, the pattern of the egg β tubulin RNAs did not change between fertilization and the early blastula stage. Then, a new 2.0-kb RNA

accumulated and became the most abundant species. The level of the largest, 2.25-kb mRNA found in eggs and early embryos decreased at the hatched blastula stage and then started to increase again at the mesenchyme blastula stage without ever becoming as prominent as the corresponding early sequence. The rise in this 2.25-kb late embryonic species was better revealed by the genomic DNA probe G-p β 6E2 than by the cDNA probe p β 2. This rise temporally paralleled the accumulation of the largest (3.4-3.6 kb) α -tubulin RNAs. The smaller 1.8-kb β -tubulin RNA remained at a low, fairly constant level up to the pluteus stage.

The amount of total β -tubulin RNA increased 2 to 11 times in late embryos (Table 1). These numbers (6-11× for p β 2 and 2-3.5× for G-p β 6E2) are rough approximations, since each of the two probes contains, in addition to cross-hybridizing coding sequences, different noncoding sequences. The noncoding sequences in p β 2 are specific for the late embryonic 2.0-kb RNA, while those in G-p β 6E2 are specific for the early and late 2.25-kb RNAs (see below). However, these results indicate that the overall levels of both α - and β -tubulin RNAs which began to rise at the blastula stage increased by similar amounts during the next several hours.

Polyadenylation of α - and β -Tubulin RNAs in Development

We have previously found that portions of all of the different size classes of α - and β -tubulin RNAs in the egg are polyadenylated. Moreover, none of these distinct size classes of RNAs are derived from another by differential polyadenylation (Alexandraki and Ruder-



FIG. 2. Autoradiograms of blotted β -tubulin RNAs from eggs and embryos at different stages of development. RNA blots similar to those presented in Figs. 1A–D were hybridized with G-p β 6E2 (A–D) and p β 2 (B') DNA probes. Panels E and F contain the same RNA samples shown in Figs. 1E and F hybridized with G-p β 6E2 probe.

man, 1985). Similarly, we found that a portion of all the α - and β -tubulin RNAs in early and late embryos were polyadenylated. Panels E and F of Figs. 1 and 2 show the poly(A)-containing fractions of RNA isolated at several different embryonic stages. Just as was seen with egg RNA, the relative hybridization intensities of some RNAs were different in total RNA and $poly(A)^+$ RNA samples. For example, when compared to total RNA, the early embryo $poly(A)^+$ RNA contained a relatively lower amount of the large (2.9-3.6 kb) α tubulin RNAs, and the late embryos $poly(A)^+$ RNA was enriched in the 2.0-kb β RNA. Comparisons of RNA sizes before and after their poly(A) tracts had been removed using RNase H indicated that none of the newly appearing RNA sizes classes arose by addition or removal of poly(A) to preexisting RNAs (data not shown).

Recruitment of α - and β -Tubulin RNAs onto Polysomes during Embryonic Development

We have previously shown that most of the α - and both β -tubulin transcripts in the egg sediment as free ribonucleoprotein particles (RNPs). One or two rare α RNAs (detectable only in highly overloaded samples of total RNA) ranging from 3.6 to 4.8 kb in length, depending on the female, are preferentially associated with small polysomes and very heavy RNP structures. Within 30 min after fertilization portions of all α - and β -tubulin RNA size classes are recruited onto polysomes (Alexandraki and Ruderman, 1985). Here, we have examined the translational activity of these maternal transcripts during cleavage and the utilization of newly accumulated RNAs after the blastula stage (Figs. 3-5).



FIG. 3. Density-gradient centrifugation profiles of α -tubulin RNAs in cytoplasmic preparations of eggs and embryos. RNA was isolated from seven fractions of each sucrose gradient, gel-blotted on nitrocellulose filters, and hybridized with ³²P-labeled G-p α 21B2 DNA. RNA isolated from the pelleted material in each gradient (P) and RNA extracted from aliquots of noncentrifuged material (T) were also analyzed. The different gradients contained similar but variable amounts of cytoplasmic samples. The discrepancy in the migration rates of the RNA samples in each panel was due to their greatly different RNA contents. The fraction collected first in each gradient (bottom) contained a smaller aliquot of centrifuged material relative to the other fractions. The panels represent the RNAs from unfertilized eggs (egg) of the same maternal origin as embryos in panels indicated by open or closed squares, respectively; 30-min zygotes (30 min); 105-min 2-cell embryos (2c); 4.5-hr 16-cell embryos (16c); 7.5-hr morulae (m); 11.5-hr blastulae (b); 16-hr hatched blastulae (hb); 20-hr mesenchyme blastulae (mb); 25-hr early gastrulae (eg); and 44-hr prisms (pr). Embryos were cultured at 16°C. Each culture derived from eggs of a single female sea urchin.

Cultures made from eggs of two different females (indicated by open and closed squares) were used to span development from the egg to the prism stage. Postmitochondrial 12,000g supernatants from several stages were fractionated on high-salt sucrose gradients into polysomal and free RNP regions. RNAs were extracted from each of the seven gradient fractions and analyzed by blot hybridization with α - or β -tubulin DNA probes.

As shown in Fig. 3, a small portion of each of the stored α -tubulin RNAs was found on polysomes within 30 min after fertilization. The extent of this recruitment increased gradually as development proceeded. By the two-cell stage about 30% of all α mRNAs were found in polysomes. All of the largest α RNA sequences (4.2 kb in the preparation shown here) appeared on polysomes at this early stage. By the morula stage the majority of all α RNAs were found in polysomes. The low-abundance, 1.75-kb α RNA was an exception to this: up to the morula stage, most of this RNA stayed in the free RNP region, suggesting that it remained mostly untranslated during early cleavage. Then, at the blastula stage, the amount of the 1.75-kb α RNA (or a new one comigrating with the early 1.75-kb RNA) rose dramatically (see also Fig. 1). Most of the new 1.75-kb α RNA was loaded onto polysomes at the blastula stage, as were the other α RNAs. Later, between the hatched blastula stage and the prism stage, a smaller fraction of the 1.75-kb α RNA was on polysomes. During this same interval, some of the larger α RNAs sedimented mainly in either the polysome or the free RNP region of the gradients. For example, in hatched and mesenchyme blastula a 2.9kb RNA was found mostly in the free RNP region, while the 3.2- and 3.6-kb RNAs were seen mostly in the polysome region. At the early gastrula and prism stages most of the 2.4- and 3.2-kb RNAs cosedimented with free RNPs.

The actual association of the RNAs with polysomes and not with heavy RNP particles was tested using two complementary experiments: treatment of the samples prior to centrifugation with puromycin, which causes the specific release of mRNAs from polysomes, and with EDTA, which causes the disruption of the ribosomal subunits and mRNA as well as RNPs and aggregates. The results of these treatments for the 16cell and mesenchyme blastula samples, shown in Fig. 5, indicated that virtually all of the α -tubulin RNAs that sedimented in the polysomal regions of the gradients were actually engaged in translation.

The translational activities of the β -tubulin RNAs during early development were similarly analyzed, using the same gradient fractions that were used for the α RNA characterizations (Figs. 4 and 5). Both of the maternal β -tubulin mRNAs were progressively recruited on polysomes after fertilization just as seen for the α -tubulin mRNAs. By the morula stage most of the 2.25-kb β mRNA was associated with polysomes; in contrast, some of the 1.8-kb β mRNA remained nonpolysomal at this stage. As shown earlier (Fig. 2), a new 2.0-kb β RNA began to accumulate at the midblastula stage. Figure 4 shows that most of this new



FIG. 4. Density-gradient centrifugation profiles of β -tubulin RNAs in cytoplasmic supernatant fractions of eggs and selected stages of embryos. RNA blots of the samples shown in Fig. 3 were hybridized with ³²P-labeled G-p β 6E2 DNA.



FIG. 5. Association of α - and β -tubulin mRNAs with polysomes in early and late embryos. RNA was isolated from seven fractions of sucrose gradients following centrifugation of the cytoplasmic preparations from the 4.5-hr (16c) and 20-hr (mb) embryos described in Fig. 3 and probes with G-p α 21B2 and G-p β 6E2 DNAs (control). Aliquots of the same cytoplasmic preparations were treated with puromycin and EDTA prior to centrifugation and further analyzed in parallel with the control samples.

2.0-kb β RNA immediately entered polysomes, and most of it continued to be associated with polysomes at later stages. A somewhat smaller fraction of the 2.25-kb β RNA was on polysomes at later stages. The rare, 1.8-kb RNA was not resolved well in the lateembryo samples. In some cases, as in the mesenchyme blastula, it can be seen throughout the gradient.

Stage- and Tissue-Specific α - and β -Tubulin mRNAs

Since all of the multiple size classes of α - and β tubulin RNAs were translationally active *in vivo*, they must represent different or overlapping mature messenger RNAs rather than various precursor forms of a single mRNA. In this section we describe the correlation of some of the different mRNA size classes with different gene sequences using the following cloned DNAs: the 3' untranslated portions of the testis cDNA clones p α 2, p β 1, p β 2, and p β 3, and the subcloned DNA fragments of α (G-p α 21B2) and β (G-p β 6E2) tubulin genomic clones.

Figure 6 shows that the α genomic DNA probe (lanes 1) and the whole α cDNA clone p α 2 (lanes 2) hybridized to the same sets of DNA fragments of total genomic DNA digested with three different restriction enzymes:

BglII, EcoRI, and XhoI. This result indicates that the 1.5-kb α genomic probe contains only α -tubulin gene sequences. Thus, any RNAs homologous to the genomic probe must represent only α -tubulin RNAs.

DNA sequence comparisons showed that G-p α 21B2 and p α 2 differ by ~15% in the coding region, with most differences residing near the 3' end. The 3' untranslated sequence of p α 2 and at least the first 250 nucleotides of 3' noncoding sequence in G-p α 21B2 are completely different (data not shown). This result indicates that the two probes represent sequences of two different genes. In agreement with this, the 3' untranslated portion of p α 2, p α 2-3', hybridized to one or two tubulin DNA fragments in the three different digestions of sea urchin genomic DNA (Fig. 6, lanes 3); these p α 2-3'-specific fragments were different from those which hybridized more intensely with G-p α 21B2 probe (lanes 1 and 2).

The two α probes, G-p α 21B2 and p α 2-3', hybridized specifically to different RNAs (Fig. 7A). The whole p α 2 probe, which contains both coding and noncoding sequences, hybridized with several RNA size classes in egg, embryo, and testis RNA. In contrast, its 3' noncoding portion hybridized only with testis RNA. There was no detectable hybridization of the p α 2-3' probe to



FIG. 6. Gel blots of sea urchin germline DNA hybridized with α - and β -tubulin coding and noncoding sequence probes. Aliquots (15 µg) of sea urchin sperm DNA were digested with restriction endonucleases (as indicated), electrophoresed in 0.8% agarose gels, transferred onto nitrocellulose filters, and hybridized with the following α - and β -tubulin cDNA and genomic DNA probes: (1) the 1.5-kb *Bgl*II DNA fragment of λ LpT21 subcloned into the *Bam*HI site of pBR322 DNA; (2) α 2; (3) the 260-bp *AluI-PstI* 3' untranslated sequence, 37 bases following the termination codon of α 2, subcloned into the *SmaI-PstI* sites of pSP64 DNA; (4) the 4.5-kb *Eco*RI DNA fragment of λ LpT6; (5) the 2.8-kb *Eco*RI DNA fragment of λ LpT6 subcloned into the *Eco*RI site of pBR322 DNA; (6) β 2; (7) the 405-base pair *MnlI-PstI* 3' untranslated sequence including 19 coding bases of β 2, subcloned into the *SmaI-PstI* sites of pSP64 DNA; (8) the 225-base pair 3' untranslated sequence of $\beta\beta$ 3 which is essentially identical to the middle portion of the probe in (7) subcloned into the *PstI* sites of pSP64 DNA; (9) the 105-base pair *MnlI-PstI* 3' untranslated sequence including 19 coding bases of α 4 sequence including 19 coding bases of $\beta\beta$ 1, subcloned into the *SmaI-PstI* sites of $\beta\beta$ 1, subcloned into the *SmaI-PstI* sites of $\beta\beta$ 1, subcloned into the *SmaI-PstI* site of pBR322 DNA; (9) the 105-base pair *MnlI-PstI* 3' untranslated sequence including 19 coding bases of $\beta\beta$ 1, subcloned into the *SmaI-PstI* sites of $\beta\beta$ 1. The arrowheads indicate the DNA restriction fragments which correspond to the cloned fragments of genomic DNA. The sizes of the cloned and total DNA restricted fragments are not identical, possibly due to individual polymorphism.

any egg or embryo RNA. This result indicates that $p\alpha 2$ represents a testis-specific α -tubulin RNA.

Both G-p α 21B2 and the whole p α 2 probe contain coding sequences, as discussed earlier. Thus, both hybridized to all of the α -tubulin RNAs in egg, embryo, and testis RNA (Fig. 7A). However, when the hybridization patterns of G-p α 21B2 were compared with those of the whole p α 2 probe, we saw that G-p α 21B2 hybridized preferentially to the 3.2 to 3.6-kb RNAs in egg and early embryo (seen also in Fig. 1). Thus, the G-p α 21B2 genomic probe must contain sequences specific for these larger maternal α -tubulin mRNAs. Gp α 21B2 gave a relatively weak signal with testis RNA probably because it is not represented in this RNA and its coding sequence has significant divergence from the testis-specific α -tubulin RNA, as discussed above.

We have used the same approach to analyze the stage-specific expression of the various cloned β -tubulin DNA sequences. Earlier DNA sequence analyses showed that the partial-length p β 1 and p β 2 sequences most likely represent β -tubulin mRNAs transcribed from different genes. Their coding regions differ by 1.7% and their 3' untranslated sequences are very different. The p β 2 and p β 3 sequences are in some way related, probably corresponding to RNAs of the same or allelic genes. The coding and proximal 3' untranslated sequences of p β 2 and p β 3 are essentially identical but p β 2 contains an additional 71 bases after the corresponding polyadenylation site of p β 3 (Alexandraki and Ruderman, 1983).



FIG. 7. Gel blots of egg, embryo, and testis RNA hybridized with α - and β -tubulin coding and noncoding sequence probes. Aliquots (5 μ g) of total RNA isolated from five different egg preparations (e 1 to 5), two cultures of hatched blastulas (hb 1 and 2), single cultures of early blastulas (eb), prisms (pr) and plutei (pl) and 0.5 μ g (A and B) and 0.25 μ g (C) of poly(A)⁺ RNA prepared from pooled testes of five animals (t) were gel-blotted and hybridized with the indicated probes. A description of each probe is provided in Fig. 6. Hybridizations were carried out at 42°C in the presence of 50% formamide when whole cDNA and genomic DNA probes were used at 38% formamide for the noncoding sequence probes. HE indicates higher autoradiographic exposures of the shown samples. The molecular weights for egg and embryo RNAs are shown on the left side; those for testis RNAs are shown on the right side. These numbers do not align because tubulin RNAs migrate differently in poly(A)⁺ RNA and in total RNA samples.

Since we do not have any sequence data available for the subcloned genomic β -tubulin probe, G-p β 6E2, its specificity was determined as follows. Genomic DNA was digested with EcoRI, AvaI, BglII, or PvuII and hybridized with G-p β 6E2 (Fig. 6, lanes 5) and p β 2 (Fig. 7, lanes 6) probes. Since $G-p\beta 6E2$ hybridized to the same sets of DNA fragments as $p\beta 2$, G- $p\beta 6E2$ must contain only β -tubulin-specific (coding and noncoding) sequences. In the EcoRI-digested genomic DNA, G $p\beta 6E2$ hybridized to its cognate 2.8-kb fragment and to several other fragments (Fig. 6, lane 5). The $p\beta 2-3'$ (lane 7) and $p\beta 3-3'$ (lane 8) probes each hybridized to two large fragments, 9.5 and 17 kb, but not to the 2.8kb DNA. This result indicates that G-p β 6E2 and p β 2/ $p\beta 3$ represent different genes. Consistent with this, the hybridization patterns obtained with other restriction enzyme digests (AvaI, BglII, and PvuII) showed that the $p\beta 2-3'$ and $p\beta 3-3'$ probes hybridized to fragments different from those hybridized most intensely by the G-p β 6E2 probe.

The $p\beta 1$ -3' probe generated a diffuse pattern with *Eco*RI-digested DNA (Fig. 6, lane 9), making it difficult to use these digests for comparisons. (This diffuse pattern might be due to nonspecific hybridization because of its small size (83 bases) or to the presence of some repetitive sequence.) In hybridizations with DNA digested with other enzymes (*AvaI*, *Bgl*II, and *Pvu*II),

 $p\beta 1$ -3' showed, in addition to a general diffuse hybridization, intense hybridization to at least one fragment which was in all cases different from those corresponding to the G-p $\beta 6E2$ probe and the other two cDNA probes $p\beta 2$ and $p\beta 3$. This result, taken together with the earlier sequence data, shows that $p\beta 1$ represents a distinct gene sequence. Thus, the probes G-p $\beta 6E2$, $p\beta 1$, and $p\beta 2/p\beta 3$ represent different β -tubulin genes.

These three different β -tubulin DNA probes hybridized to different RNAs, as shown in Fig. 7B. The p β 1-3' probe hybridized only to the 1.8-kb RNA in eggs and embryos, and not to the 2.25-kb RNA in early embryos or the 2.0-kb RNA in later embryos. In testis, p β 1-3' hybridized fairly well to an ~2.0 kb RNA, but gave a narrower band than did the whole p β 2 probe. The size difference between the embryo and testis p β 1-3' hybridizing RNAs indicates that these RNAs are probably homologous but not identical.

p β 2-3' hybridized well to the 2.0-kb β -tubulin mRNA which appears in late embryos; it gave a weak signal with the 1.8-kb RNA in late embryos (resolved better on other gels not shown) and a very faint signal in higher exposures (Fig. 7B, HE) of egg RNA. p β 2-3' hybridized strongly with testis RNA. As expected, p β 3-3' gave hybridization patterns that were similar to those of p β 2-3' in egg, embryo, and testis RNA samples.

These results indicate that the 1.8-kb RNA (p β 1-

specific) and the 2.0-kb RNA ($p\beta 2/p\beta 3$ -specific) in eggs and embryos are encoded by different genes. Also, because the $p\beta 1$ -3' probe hybridized to both early and late 1.8-kb RNAs, whereas the $p\beta 2$ -3' probe hybridized primarily to the late 1.8-kb RNA, the late-stage-embryo RNAs probably contained two different 1.8-kb RNAs.

The genomic probe G-p β 6E2 and the whole p β 2 probe, which both contain coding sequences, hybridized to all of the β -tubulin RNAs in eggs, embryos, and testis. However, G-p β 6E2 hybridized preferentially to the 2.25-kb β RNA present in eggs and late embryos, which suggests that it contains noncoding sequences specific for that RNA (Fig. 7B). This indicates that the 2.25-kb RNA is encoded by a third β -tubulin gene, Gp β 6E2-like, that differs from those corresponding to p β 1 and p β 2/p β 3. We do not know if G-p β 6E2 represents a functional gene or whether it contains sequences similar to an unidentified functional gene.

 λ LpT6, the parent genomic clone of the probe Gp β 6E2, contains a second β -tubulin gene sequence (Alexandraki and Ruderman, 1981). This second β sequence is present in a 4.5-kb *Eco*RI fragment called λ 6E1 (Fig. 6, lanes 4). This probe showed an even more pronounced hybridization to the 2.25-kb β -tubulin RNA than did G-p β 6E2 (Fig. 7C). This result suggests that the λ 6E1 gene may have the same temporal pattern of expression as the G-p β 6E2 gene, with which it is linked, or other, G-p β 6E2-like genes. Alternatively, it may be a pseudogene that contains noncoding sequences closely related to those of G-p β 6E2.

Thus, there are at least three different β -tubulin genes which are expressed at different times during development and produce different sizes of β -tubulin RNAs. The p β 1-specific gene hybridizes to a 1.8-kb RNA that is found in eggs and late embryos. It is also expressed in testis, where it hybridizes to an ~2.0-kb RNA. The p β 2- (and p β 3-) corresponding genes are specific for the 2.0-kb RNA that is found in late embryos; they, too, are expressed in testis. G-p β 6E2 and λ 6E1 preferentially hybridize to a 2.25-kb RNA that is present in eggs and accumulates in late embryos.

DISCUSSION

Oogenetic and Embryonic Tubulin mRNAs

Our results show that the developing sea urchin embryo makes use of two different classes of α - and β tubulin mRNAs, oogenetic or "maternal" mRNAs and newly transcribed embryonic mRNAs, and that the embryo has different translational programs for these two classes. The maternal tubulin mRNAs, made during oogenesis and accumulated in the mature unfertilized egg, consist of multiple, polymorphic transcripts. Little is known about their synthesis or translational activity during the active period of oocyte growth, except that they (or other, unidentified tubulin mRNAs) must direct the synthesis of a large pool of tubulin proteins (Cognetti et al., 1977; Raff et al., 1971). In the mature egg, which has a very low overall rate of protein synthesis, these RNAs are for the most part inactive (Alexandraki and Ruderman, 1985). The β -tubulin RNAs (1.8 and 2.25 kb) were found to be completely inactive in the egg. The situation for the α RNAs is different from that of the β RNAs and considerably more complicated. Eggs contain several different size classes of α RNAs, ranging from 1.75 to 4.8 kb, and there is significant variation among the RNA gel blot patterns of α RNAs from eggs of different individuals. The one or two largest and relatively rare RNAs (3.6-4.8 kb, depending on the female) differ from the small α RNAs (1.75–3.7 kb) in that they are present almost exclusively on polysomes in the unfertilized egg. whereas only a very small portion of each of the others is translated before fertilization.

The tubulin RNA gel blot patterns of cleavage-stage embryos up to the early blastula stage were essentially identical to those of the maternal α - and β -tubulin sequences. This result suggests that the maternal tubulin RNA sequences present in the unfertilized egg persist throughout cleavage. Since we did not measure the contributions of any new embryonic transcripts to this pool we cannot rule out the possibility that during the early cleavage divisions the maternal sequences decline and are replaced with similar amounts of identical sequences, but this seems unlikely for the following reasons. First, using transcriptional inhibitors, Raff et al. (1972) found that most of the early rise in tubulin synthesis was due to the recruitment of these maternal tubulin RNA sequences. Second, the maternal tubulin RNAs show considerable allelic polymorphism (Alexandraki and Ruderman, 1985). If the same genes were being transcribed in embryos, we would have expected to see a considerable increase in the heterogeneity of the cleavage-stage tubulin RNAs as polymorphic, electrophoretically distinct transcripts accumulated from the *paternal* genome. Since that was not the case, we conclude that there was essentially no change in the maternal tubulin RNAs during cleavage and that these RNAs were responsible for the increase in tubulin synthesis during cleavage discussed below.

Major qualitative and quantitative changes started in the early blastula about 6 to 8 hr after fertilization and led to the late embryonic (postblastula) pattern of α - and β -tubulin RNAs. At this time, the amount of the 1.75-kb α RNA (or a new one that comigrated with the maternal 1.75-kb RNA) increased and a new, 2.0kb β RNA appeared. Both of these RNAs continued to

accumulate; their levels peaked at the swimming blastula stage and remained more or less constant thereafter, varying somewhat in different cultures. These two RNAs were the most prominent ones in late-stage embryos. This rise in new transcripts was accompanied by a decline in most maternal sequences. Where they could be distinguished, it was clear that the most abundant maternal sequences began to disappear at the hatched blastula stage. Between the mesenchyme blastula and pluteus stages, qualitative and quantitative changes in the less abundant (at that time) RNAs occurred, including the appearance of new, long RNAs. The overall amounts of both α and β RNAs increased by about two- to fivefold during postblastula development. Most of this was due to the rise in the 1.75-kb α RNA and the 2.0-kb β RNA. The exact degree of the increase could not be measured very accurately because different probes recognized different mRNAs within the α - and β -tubulin RNA classes, thus giving different signals. We did not make additional measurements using only coding sequence probes.

The major shifts in the amounts and kinds of translationally active tubulin RNAs occurred at the blastula stage, a time accompanied by many other changes. The rate of cell division declines, cilia are produced, and several morphological changes occur in preparation for gastrulation. This is roughly the time when the maternal mRNA supply alone is no longer sufficient to sustain embryonic development (Gross et al., 1964). Between the blastula and gastrula stages there are prominent changes in the kinds of proteins being made as certain maternal mRNAs disappear and many new RNAs appear or are augmented (Ruderman et al., 1974; Brandhorst, 1976; Hieter et al., 1979; Crain et al., 1981; Bedard and Brandhorst, 1983; Freigen et al., 1983; Harkey and Whiteley, 1983). The shift from a complex set of several relatively low-abundance maternal tubulin RNA sequences to a simpler pattern of a single prevalent sequence plus a few less abundant species (especially seen with α RNAs) resembles the general trend toward a qualitative decrease in rare polysomal mRNAs (Davidson et al., 1982a) and a quantitative shift to the synthesis of fewer, more abundant proteins (Harkey and Whiteley, 1983). One of the best-studied examples of a transition from one set of mRNAs to another at the blastula stage is the switch from the synthesis of early to late histone variants. By the hatched blastula stage, the pool of maternal mRNAs encoding the early histones is reduced by 10-fold and newly transcribed mRNAs encoding the late variants rise and peak at the mesenchyme blastula stage (reviewed by Maxson et al., 1983). It is worth noting, however, that not all RNAs show large changes at this stage. Several maternal mRNAs are known to persist beyond the blastula stage (Tufaro and Brandhorst, 1982) and the levels of many abundant RNAs do not change significantly between the egg and pluteus stage (Flytzanis *et al.*, 1982; Freigen *et al.*, 1983).

Translational Recruitment of Maternal and Embryonic Tubulin mRNAs

As pointed out earlier, none of the maternal β tubulin mRNAs are detected on polysomes in the unfertilized egg, whereas a part of each of the α RNAs is active at that stage. Within 30 min of fertilization, a small portion of all stored α and β RNAs moves into the polysomes (Alexandraki and Ruderman, 1985). Experiments presented here showed that, in general, more and more maternal α - and β -tubulin mRNAs were recruited into polysomes as development progressed until, at the morula stage, almost all of the maternal sequences had been translocated from the inactive RNP compartment to the active polysome compartment. This overall increase in the translational activities of individual α - and β -tubulin mRNAs agrees well with the postfertilization rise in tubulin protein synthesis previously described by Raff et al. (1971) and Raff (1975).

It is difficult to say from these data whether the activation of tubulin mRNAs exactly parallels the large increase in the overall rate of protein synthesis that occurs within a few minutes of fertilization and continues to increase up to the two-cell stage, as has been reported by Wells et al. (1981). Tubulin mRNA recruitment appeared to follow the overall, gradual rise in the rate of protein synthesis that occurs between the two-cell and blastula stages (Goustin and Wilt, 1981). However, not all maternal tubulin mRNAs were marshaled equally. The activation of the smallest, 1.75kb α RNA and 1.8-kb β RNA lagged behind the others. and at the morula stage, when most maternal tubulin mRNAs were found on polysomes, significant portions of these two mRNAs remained in the inactive RNP compartment.

The kinetics of tubulin RNA recruitment differs significantly from those of the histone mRNAs, the only other ones that have been examined in detail. The maternal histone RNAs are sequestered in the egg pronucleus (Showman *et al.*, 1982; DeLeon *et al.*, 1983) and remain there until the time of the first cleavage division (about 90 min) when they are dispersed from the nucleus. Then, they move rapidly into polysomes and, in the 4-hr embryo, more than 85% of these RNAs are translationally active (Wells *et al.*, 1981). Clearly, comparisons of these data indicate that different maternal mRNAs have different temporal programs of activation. Unlike the gradual mobilization of most maternal tubulin mRNAs between fertilization and the morula stage, almost all of the prominent, new embryonic transcripts (the 1.75-kb α and 2.0-kb β RNAs) were recruited onto blastula polysomes without any significant delay. At later stages a portion of the 1.75-kb α RNA and variable amounts of some of the other, less abundant α and β mRNAs were found in the RNP compartment, indicating that translational choices continue to be made in late-stage embryos.

Harkey and Whiteley (1983) found a coordinate increase in the amounts of α - and β -tubulins synthesized in the postblastula embryos. Our data show that this is due to the rise of α and β RNA levels. We noticed, however, that different percentages of α and β RNAs were on polysomes at the late embryos. This suggests that the final coordination in the amounts of α - and β -tubulins may be regulated differently for the two protein classes.

Different Stage- and Tissue-Specific Tubulin mRNAs

The presence of multiple tubulin gene sequences in the sea urchin genome (Alexandraki and Ruderman, 1981, and this paper) and multiple tubulin mRNA sequences (Alexandraki and Ruderman, 1983, 1985, and this paper) suggests that there are different functional genes which give rise to different functional tubulin mRNAs. These mRNAs might, in turn, direct the synthesis of some of the tubulin protein variants that have been observed (Stephens, 1978; Asai et al., 1982; Dietrich and Wilson, 1983; Suprenant and Rebhun, 1983). On the other hand, some of the tubulin gene sequences might be pseudogenes, as is the case for many of the tubulin sequences in rat and human genomes (Lemischka and Sharp, 1982; Lee et al., 1983). Heterogeneous mRNAs might arise by differential splicing of a single gene transcript. The electrophoretic and antigenic variations in tubulin proteins could represent post-translational modifications. Indeed, organisms like *Drosophila* and chicken get by with just four α - and four β -tubulin genes (Raff *et al.*, 1982; Kalfayan and Wensink, 1982; Lopata et al., 1983).

To begin to sort out some of these possibilities, we compared the hybridization patterns of various subcloned fragments of some of our cDNA and genomic clones to egg, embryo, and testis RNAs. The results of these comparisons argue that at least two different α genes and three different β genes are functional and are differentially expressed during development. The 3' noncoding region of the cDNA clone p α 2 recognized only testis-specific RNA. The genomic subclone Gp α 21B2 hybridized preferentially to an egg-specific α tubulin mRNA whose size was 3.2-3.6 kb, depending on the individual female. At this point we do not know whether the other α RNAs in the egg and embryos derive from one or a few genes by alternative splicing or if each α RNA derives from a different gene. Considering the observed individual polymorphism of α RNAs it is almost certain that some of the differentsize RNAs derive from allelic polymorphic genes.

The 3' untranslated region of the cDNA clone $p\beta 2$ hybridized to the embryonic 2.0-kb RNA, which first appeared at the blastula stage, but not to the two egg β RNAs. However, its expression was not confined exclusively to embryonic stages since it was detected in testis RNA as well. The genomic probe G-p β 6E2 hybridized preferentially to the 2.25-kb RNA found in eggs and in late-stage embryos. The $p\beta 1-3'$ noncoding probe hybridized to the 1.8-kb RNA found in eggs and late embryos, and it was detected in a 2.0-kb testis RNA as well. It is possible that each RNA size class contains several different sequences. Our data suggest that this may be true for the late 1.8-kb RNA. A second complication of these results is that the probes used could be hybridizing to transcripts of their cognate genes or to those of other similar, but not identical. genes. Subgroups of genes containing similar noncoding sequences are commonly found in the analysis of multigene families.

These results make it clear that there are different tubulin mRNAs that are expressed in a stage- and tissue-specific manner during sea urchin development, but they say nothing about the diversity of the proteins encoded by these mRNAs. There is evidence that sequence heterogeneity in members of multigene families may not reflect specific functional requirements of their coresponding proteins but instead may result from the organization of genes into developmentally specific units in the genome (Davidson *et al.*, 1982b; Kemphues *et al.*, 1982). Therefore it remains to be found if the various urchin tubulin RNAs correspond to different organelle-specific or stage-specific tubulins.

Much of the tubulin required for mitotic spindles and cilia is already present in the egg. The tubulins that are newly synthesized by the early embryo could be used for these same structures or they could be stored for later functions. As development progresses and different lineages become established, different tissues express different amounts, and possibly different types of tubulins. The micromere lineage produces relatively low amounts of tubulins, whereas the epithelial cells of the ectodermal lineage synthesize tubulins at a very high rate (Harkey and Whiteley, 1982, 1983). One possibility is that the very abundant lateembryonic α and β RNAs are involved in mitosis and ciliagenesis, while the minor ones might encode the cytoplasmic tubulins involved in morphogenetic changes or in the differentiation of the primary mesenchyme cells of the micromere lineage.

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